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NEUTROPHIL ACTIVATING FACTOR (NAF) INDUCES POLYMORPHONUCLEAR LEUKOCYTE ADHERENCE TO ENDOTHELIAL CELLS AND TO SUBENDOTHELIAL MATRIX PROTEINS

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Neutrophil activating factor is a polypeptide cytokine released from stimulated mononuclear phagocytes and endothelial cells. We found that neutrophil activating factor induced time- and concentration-dependent binding of human polymorphonuclear leukocytes to endothelial monolayers and subendothelial matrix proteins, via a mechanism that involves altered expression of the leukocyte CD_{11}/CD_{18} glycoproteins. Thus, neutrophil activating factor is a third mediator, in addition to platelet-activating factor and endothelial leukocyte adhesion molecule 1, that is synthesized by activated endothelium and that can induce polymorphonuclear leukocyte adhesion to endothelial cells. Because NAF is released into the pericellular fluid, it may also stimulate binding of the leukocytes to exposed subendothelial structures at sites of vascular injury.

Neutrophil-activating factor (NAF), a 72 amino acid polypeptide, was originally isolated from human mononuclear phagocytes stimulated with bacterial lipopolysaccharide (1,2). A gene coding for NAF was subsequently expressed in E. coli and a recombinant protein that is equivalent to naturally-occurring NAF was produced (3). NAF is identical to monocyte-derived neutrophil chemotactic factor (MDNCF) isolated by Yoshimura *et al* (4-5) and to monocyte-derived peptides described by others (6-7). The polypeptide is different from interleukin-1α and β and from tumor necrosis factor α and β, but has remarkable sequence homology with members of a family of small proteins with diverse biologic activities, including platelet basic protein and its cleavage products, platelet factor 4, and certain other peptides (1,5,7,8). NAF induces chemotaxis, degranulation, and respiratory burst

ABBREVIATIONS: NAF, Neutrophil activating factor; EC, Endothelial cells; PMNs, Polymorphonuclear leukocytes; HUVEC, Human umbilical vein endothelial cells; LAD, leukocyte adhesion deficiency; ELAM-1, Endothelial leukocyte adhesion molecule one; PAF, platelet-activating factor.

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activity in human polymorphonuclear leukocytes (PMN) at nanomolar concentations by interacting with a specific plasma membrane receptor (2,3). It induces a signal transduction process similar to that initiated by other chemotactic agonists (9). Recently it was reported that NAF or NAF truncation variants are synthesized and released by cytokine-activated human endothelial cells (EC) (10-12); the regulation of its synthesis in EC appears to be at the level of transcription (10,12). In this study we show that NAF induces PMN adhesion to human EC and to subendothelial matrix proteins by a mechanism that involves enhanced expression of CD_{11}/CD_{18} integrins. Thus, NAF released from activated EC may influence PMN binding to the vessel wall in inflammatory conditions.

MATERIALS AND METHODS

NAF: Recombinant human NAF prepared as described (3) was used in these experiments. Recombinant NAF has previously been shown to have the same activity and potency as natural NAF in assays of chemotaxis, respiratory burst activity, and degranulation using human PMNs (3).

PMN ADHERENCE TO EC: Suspensions of isolated PMNs radiolabeled with ^{111}Indium oxine and primary monolayers of tightly confluent human umbilical vein EC were prepared as described (13,14). The details of the adhesion assay have been published (13,14). Briefly, the culture medium was removed from EC monolayers, they were washed once with buffer, and ^{111}In-labeled PMNs in Hanks Balanced Salt Solution containing 0.5% human albumin ($5.5x10^6$ PMNs/ml) were layered over them. Control buffer, NAF, or another agonist was added to the PMN suspension, and the PMNs and EC were incubated at 37° in a humidified atmosphere of 5% CO_2, 95% air for the indicated times. The number of adherent PMNs was then determined as described and is expressed as a percent of the number added (13). In some experiments PMNs were treated with control buffer, NAF, or monoclonal antibodies prior to addition to EC or matrices (see below).

PMN BINDING TO SUBENDOTHELIAL MATRICES AND PURIFIED PROTEIN MATRICES: Subendothelial matrices were prepared as described (15). We previously confirmed that such matrices contain vonWillebrand Factor, fibronectin, and vitronectin (Parker CJ, Zimmerman GA, unpublished observation) and others have shown that subendothelial matrices include laminin, Type IV collagen, and other proteins (reviewed in 16). Protein matrices of gelatin (Type A, Fisher Scientific, Co.), fibronectin, or laminin (Collaborative Research Inc) were prepared as described (15). The PMN adhesion assay (15) was similar to that for EC (see Legend to Table I, and Figure 2).

ROLE OF LEUKOCYTE CD_{11}/CD_{18} INTEGRINS: PMNs were pretreated with IB4 (10μg/ml), an IgG_{2a} murine monoclonal antibody (17) against the β chain (CD_{18}) of the leukocyte CD_{11}/CD_{18} glycoprotein integrins (18), for 10 minutes at room temperature prior to addition to EC monolayers or protein matrices. IB4 was generously provided by Samuel Wright (Rockefeller University, New York, N.Y.) and was shown to immunoprecipitate each of the three CD_{11}/CD_{18} heterodimers, indicating that it identifies an epitope on the common β chain (17). W6/32, an isotype-matched murine monoclonal antibody against an HLA Class I determinant (American Type Culture Collection, Rockville, MD), was used as a control. The

response to NAF of PMNs isolated from a patient with leukocyte adhesion deficiency ("LAD") (18) was measured in one experiment (Table I).

RESULTS AND DISCUSSION

NAF stimulated PMNs to adhere to cultured human EC monolayers. In 4 experiments the adhesion of PMNs in response to control buffer was 12 ± 5% after a 10 minute incubation with EC, whereas the adhesion of PMNs stimulated with NAF (10^{-7}M) was 34 ± 5% (Figure 1). The NAF-induced PMN adhesion to EC was concentration-dependent, with a threshold at 10^{-9} - 10^{-8}M and a maximal effect at 10^{-6}M NAF. Pretreatment of PMNs with NAF for brief periods (1 or 5 minutes), followed by addition of the PMNs to EC monolayers and incubation for a further 5 minutes, resulted in enhanced PMN adhesion to the EC. Addition of NAF to PMNs overlying gelatin matrices also induced binding of PMNs. The adhesion of PMNs to matrices in response to control buffer and 10^{-7}M NAF was 10 ± 5% and 47 ± 3.5%, respectively, after a 10 minute incubation (n = 3). These results demonstrate that NAF directly induces PMN adhesiveness. The enhanced binding was completely inhibited in calcium and magnesium-deficient buffer, indicating that divalent cations are required. At equivalent molar concentrations, the adhesion to EC or to protein matrices stimulated by NAF was equal to, or less than, that induced by n-formylmethionyl-leucyl-phenylalanine (fMLP) (Table I and data not shown).

NAF stimulated PMNs to bind to complex subendothelial matrices and to confluent EC monolayers in a time-dependent fashion. When NAF was added to PMNs overlying subendothelial matrices, adhesion occurred rapidly, was maximal at

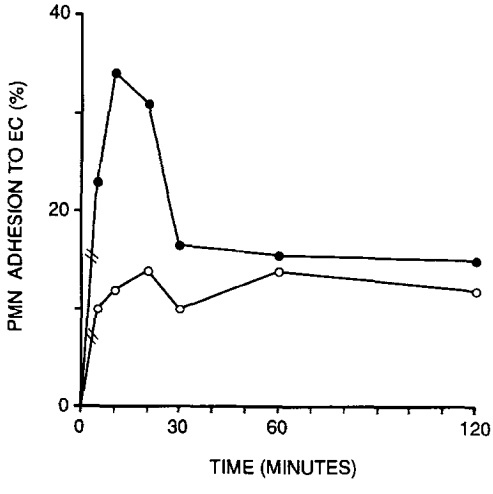

FIGURE 1: NAF stimulates PMN adhesion to EC monolayers. NAF or control buffer was added to PMN suspensions overlying EC monolayers, the cells were incubated together for the indicated times, and the fraction of adherent PMNs was determined as described in "Methods". Closed circles, NAF; open circles, control buffer. The figure summarizes the results from 4 experiments (different PMN donors and EC cultures) with a minimum of 2 determinations for each point.

TABLE I: EFFECT OF ANTI-CD_{11}/CD_{18} ANTIBODIES AND CD_{11}/CD_{18} DEFICIENCY ON NAF-STIMULATED PMN BINDING

EXPERIMENT	AGONIST	ADHESION "TARGET"	PMN CONDITIONS	TIME OF INCUBATION	% BINDING	% INHIBITION
I	Buffer	HUVEC	Buffer	10'	9%	-
	NAF,10^{-7}M	"	W6/32	"	22±2%	-
	"	"	IB4	"	10±1%	92%
II	Buffer	Gelatin Matrix	Buffer	5'	4%	-
	NAF,10^{-7}M	"	W6/32	"	21%	-
	"	"	IB4	"	4%	100%
III	Buffer	Gelatin Matrix	Control PMNs	5'	3%	-
	NAF,10^{-7}M	"	"	"	22%	-
	fMLP,10^{-7}M	"	"	"	35%	-
	Buffer	"	"LAD" PMNs	"	3%	-
	NAF,10^{-7}M	"	"	"	3%	100%
	fMLP,10^{-7}M	"	"	"	3%	100%
IV	Buffer	Laminin Matrix	Buffer	5'	7±0.5%	-
	NAF,10^{-6}M	"	"	"	60±0.5%	-
	"	"	IB4	"	13±1%	89%
	Buffer	"	Buffer	30'	9±0.5%	-
	NAF,10^{-6}M	"	Buffer	"	52±2%	-
	"	"	IB4	"	9±1%	100%

^{111}In-labeled PMNs were added to human umbilical vein EC monolayers (HUVEC) or culture wells coated with gelatin or laminin, the agonist was added, and PMN binding was measured after the indicated time. Values for "% binding" indicate the results of single determinations or mean ± standard deviation of duplicate or triplicate measurements in each experiment. PMNs were pretreated with control buffer, the anti-CD18 monoclonal antibody, IB4 (10μg/ml), or an isotype-matched control antibody, W6/32 (10μg/ml), for 10' at room temperature prior to addition to the "target" surfaces. In experiments I and II, the NAF-stimulated adhesion of PMNs pretreated with W6/32 was the same as, or slightly greater than, that of PMNs pretreated with control buffer alone. "LAD" PMNs were isolated from a patient with CD_{11}/CD_{18} deficiency. The characteristics of this patient will be described in detail elsewhere (Bohnsack J, manuscript in preparation). "Control PMNs", and PMNs used in the experiments with monoclonal antibodies, were isolated from normal adult donors. In two additional experiments, IB4 completely blocked NAF-stimulated PMN binding to gelatin matrices at 5', and blocked binding to gelatin matrices after a 30' incubation by 22% and 100%.

10-20 minutes and persisted at maximal levels for 30 minutes, and declined slowly over the remainder of the 2 hour assay period (Figure 2). The kinetics of binding were similar when laminin, gelatin, or fibronectin matrices were used (Table I and data not shown). This contrasts with the time course of NAF-induced PMN adhesion to endothelium. NAF-stimulated adhesion to EC monolayers was also rapid, with a peak at 10 minutes, but the binding was rapidly reversible with a return to near control values by 30 minutes (Figure 1). This result indicates that

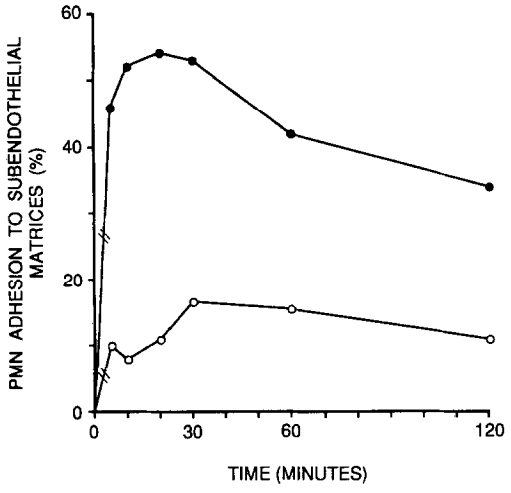

FIGURE 2: NAF stimulates PMN adhesion to subendotnelial matrices. NAF or control buffer was added to PMN suspensions overlying subendothelial matrices. After incubation for the indicated times, the binding of PMNs to the matrix proteins was determined (Methods). The figure indicates the results from 2 experiments with different PMN donors and matrices from different EC isolates.

NAF-induced adhesion of PMNs to EC and to subendothelial matrices is differentially regulated, perhaps by agents generated by the EC (19,20). The latter possibility is suggested by an experiment in which PMNs that were preincubated in suspension with 10^{-7}M NAF for 5-20' in the absence of EC, and then were added to EC for a fixed 5' incubation period, adhered to the EC monolayers to a similar extent regardless of the time of preincubation with NAF (not shown).

The mechanism of NAF-stimulated PMN binding to EC and to subcellular proteins involves increased expression of the leukocyte CD_{11}/CD_{18} integrins. This is demonstrated by the experiments in Table I, in which a monoclonal antibody to CD_{18} inhibited PMN binding to EC, and to protein matrices. Furthermore, PMNs from a patient with congenital absence of CD_{11}/CD_{18} failed to adhere in response to NAF. These findings are consistent with observations that NAF enhances the expression of CD_{11b}/CD_{18} on human PMNs (21).

Two well-characterized molecules that induce PMN binding to EC, platelet-activating factor ("PAF"; 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (13) and endothelial leukocyte adhesion molecule 1 ("ELAM-1") (22), have been shown to be synthesized by activated endothelium (23,24). The current results indicate that NAF is a third mediator of adhesion that is synthesized by EC when they are appropriately stimulated. In contrast to PAF and ELAM-1, which are retained by the EC (25,26), NAF is released into the pericellular fluid (10-12). Thus, NAF may promote the adhesion of activated PMNs (2,3,9) to areas of exposed subendothelial matrix adjacent to stimulated EC at sites of vascular injury, in addition to mediating binding of PMNs to the endothelium. It is unknown if some of the NAF remains in the plasma membranes of EC or mononuclear cells, and is biologically active in this position, as

reported for other cytokines (27,28). The expression of NAF by EC is slower that that of PAF and ELAM-1, requiring hours for induction and 24 hours to reach its maximum (12). Thus, the activated or injured intima appears to have a repertoire of biologically-active molecules that can be expressed in a time-dependent fashion and act by different mechanisms to influence the adhesion and activation of PMNs.

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